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The DNA replication factor RPA physically associates with the tumor suppressor protein p53, an interaction that could be important for the function of both these proteins in normal and cancer cells. We have made small deletion fragments of the p53 protein and expressed them as GST-fusion proteins. These fragments showed that RPA binds to point mutations in p53. Two such mutant forms of p53 have the desired property of not binding RPA. Both these mutant versions of p53 retain wild type transcriptional activation property. Thus we are now in the position to use these mutant versions of p53 to test whether the RPA binding ability of p53 is important for its multiple functions. Deletion fragments of Rpa1 were made by in vitro transcription translation to determine which parts of Rpa1 bound to p53. Binding to p53 requires a region of Rpa1 overlapping with the high affinity binding site for DNA explaining how p53 could competitively interfere with the DNA binding function of RPA. The evolutionarily conserved putative zinc finger near the C terminus of Rpa1 was not required for binding to p53.

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This work is being done in concert with a postdoctoral fellow in my laboratory, Dr. Junjie Chen, who is independently supported by a postdoctoral fellowship: DAMD17-94-J-4070. He is revising his report to ensure that it is significantly different from this one. However, there is significant overlap in the work described because it is being done in concert by a postdoctoral fellow (Dr. Junjie Chen) and a principal investigator (Dr. Anindya Dutta). The grants support our separate salaries.

INTRODUCTION

Importance of p53 function in breast cancer: The p53 protein is an important tumor suppressor which is inactivated by mutation in up to 50% of breast cancers [1-4]. The mutated p53 is often over-expressed, so that intense staining with anti p53 antibodies upon immunohistochemistry of breast cancers has been used as a marker for such inactivation. When this issue was examined directly, 100% correlation was found in 15 patients between p53 accumulation and p53 mutation [5] Cytogenetic, and DNA analysis techniques have detected loss of heterozygosity of the p53 locus (17p13.1) in up to 64% of primary breast cancers [6]. Such a loss of heterozygosity is also considered a marker for inactivation of a tumor suppressor gene, because the remaining allele of the gene usually harbors an inactivating point mutation. Direct analysis of the p53 gene by PCR amplification of tumor DNA has shown mutations in 32% of invasive and 12% of intraductal/predominantly intraductal breast carcinomas, implying that mutation of the p53 gene is important for breast cancer progression [7]. Over-expression of p53 in breast cancer has been associated with increased proliferation: the % of cells in S phase being 7.1% in p53 positive tumors vs 4.1% in p53 negative tumors [3]. In multiple studies, mutation of the p53 gene in a breast cancer is associated with shorter disease free interval and decreased overall survival, independent of the presence of axillary node metastases [3, 4, 10, 11]. In one study, for example, immunohistochemistry on paraffin sections in 289 axillary node negative breast cancer cases showed increased levels of p53 in 41 patients. p53 positive tumors were associated with an eight year survival of 56% as opposed to 81% for p53 negative tumors [11].

In a small group of familial breast cancer patients (with Li Fraumeni syndrome), inactivating germ-line mutations are seen in the p53 gene, indicating the importance of normal p53 in preventing the appearance and progression of breast cancers [12, 13]. Cells from patients with Li-Fraumeni syndrome are heterozygous (mutant/wild type) for p53 in somatic non-neoplastic tissue, and homozygous for mutant p53 in tumor tissues. However, germ-line mutation of p53 is not a common cause of familial breast cancers [14]. Finally, the introduction of wild type p53 into breast cancer cell lines with mutant p53 reversed their ability to form colonies in soft agar and produce tumors in nude mice [15]. This indicates that restoring normal p53 (or some of the functions carried out by p53) to breast cancers could have therapeutic value.

Finally, in a study of 27 cases of inflammatory breast cancer showed that 30% had increased p53 in the nucleus (mutant p53), and 33% had no p53 staining [16]. The surprising result was that 37% of the tumors had wild type p53 which showed cytoplasmic staining with nuclear sparing. Therefore, beside the mutational inactivation of p53, the tumor suppressor may also be inactivated in breast cancers by post-translational processes which interfere with nuclear localization.

Beside mutation of the p53 gene, other changes in the genome seen in breast cancers include amplification of certain dominant oncogenes, e.g. erbB, erbB2, erbB3, c-myc, H-ras, hst and int2 (reviewed in [17]). Amplification of DNA sequences in cells is suppressed by wild type p53, so that the mutational inactivation of p53 may also contribute to the progression of breast cancer by allowing DNA amplification.

p53: Human p53 is a 393 amino acid containing nuclear phosphoprotein. Both the germinal and the somatic mutations of p53 which are seen in cancers appear to be "loss of function" mutations. Several DNA tumor virus oncogenes, e.g. the E6 protein of the human papilloma virus or T Antigen of SV40 virus, specifically inactivate the p53 gene by protein-protein interactions. Likewise, a dominant cellular oncogene, mdm2, overexpressed in several sarcomas, exerts its tumorigenic function by interacting with and inactivating wild type p53 protein. Protein-protein interaction could be a common mode of inactivation of p53 in tumors which do not have somatic mutations in the p53 gene. Therefore, the overexpression of a cellular protein which interacts with p53 through its functionally significant domains may be as important for the pathogenesis of breast cancer as mutation of the p53 gene.

Presence of wild type p53 in a tumor is also important for responsiveness to chemotherapy and radiotherapy. The damage to DNA produced by these therapeutic agents leads to apoptosis of the cancer cells through a mechanism that requires wild type p53. This could be the reason behind the better prognosis of tumors with wild type p53 than those with mutated p53.

Overexpression of the wild type p53 protein arrests cell growth at a specific stage in the cell cycle, just before the onset of DNA replication at the G1-S boundary. Wild type p53 is also essential for DNA repair following radiation induced DNA damage, or for apoptosis of the cell if the DNA damage is extensive. The transforming mutants of p53 are defective in all these functions. Therefore of major concern in the field of breast cancer research is how the wild type p53 protein carries out these three diverse functions (inhibition of S phase, pause in DNA replication to repair damaged DNA, inhibition of DNA amplification). A simple hypothesis would be that p53 executes these diverse functions by directly inhibiting the DNA replication machinery. A clear understanding of the mechanism of action of p53 will allow (a) better diagnostic and prognostic tests for breast cancers, and (b) the design of a therapeutic strategy which restores p53 function in cancers with mutant p53.

Three mechanisms have been proposed by which p53 inhibits DNA replication. p53 has a sequence-specific DNA binding activity and an N terminal domain that behaves like a transcriptional activation domain. Indeed the p53 binding sites present upstream from a muscle creatinine kinase promoter acts as a transcriptional enhancer in co-transfection experiments with plasmids expressing wild-type p53. Therefore one mechanism by which p53 could act as a suppressor of S phase is by the transcriptional induction of genes that negatively regulate cell growth. However p53 can also bind to a basal transcriptional factor, the TATA box binding protein (TBP), through the N terminal transcription activation domain, and inhibit basal transcription from TATA box containing promoters . This has led to the second suggestion: p53 inhibits the expression of key S phase activators. p53 also interacts with the SV40 TAg through the middle half of the p53 protein (p53 was discovered because of this interaction). Through this physical interaction p53 inhibits the helicase activity of T antigen and inhibits the SV40 based *in vitro* DNA replication. Therefore the third possibility is that p53 directly interacts and inhibits a cellular origin binding protein replication initiator protein (as yet unidentified).

p53 physically interacts with and inhibits the function of the DNA replication factor RPA (described in the next paragraph) [18]. Thus p53 could directly interact with and regulate the DNA replication machinery. RPA binds to two separate domains of p53: an N-terminal transcriptional activation domain , and a C terminal domain that overlaps with the nuclear localization signal.

RPA: RPA (RF-A or human ssb; RPA stands for replication protein A) is a complex of three polypeptides of 70 kD, 34 kD and 13 kD, essential for SV40 DNA replication *in vitro* and excision repair in animal cells. The 70 kD subunit from human cells binds to single stranded DNA, and supports unwinding of the SV40 ori, but is

unable to support SV40 DNA replication *in vitro*, implying that the 34 and 13 kD subunits execute a function essential to DNA replication. Monoclonal antibodies against the 34 kD subunit inhibit DNA replication *in vitro*, again suggesting that the holocomplex RPA carries out functions essential to DNA replication through the 34 kD subunit. In *S. cerevisiae* the genes for the 70, 34 and 13 kD subunits are each essential for viability. The individual 70, 34 and 13 kD protein subunits are referred to as Rpa1, Rpa2 and Rpa3, respectively, and the genes are referred to as *Rpa1*, *Rpa2* and *Rpa3*.

Regulation of RPA by direct interaction with p53. As mentioned above, we have recently discovered that p53 physically interacts with and inhibits the DNA binding activity of RPA. This suggests a novel mechanism by which p53 inhibits DNA replication and executes the diverse activities which are lost in breast cancers (growth repression, pause in DNA replication for repair of damaged DNA and repression of DNA amplification).

Most of the transforming mutants of p53 fail to inhibit DNA replication *in vivo* and *in vitro* and fail to bind T antigen or to the specific p53 binding DNA element. However, the two transforming mutants of p53 we examined (R175H and R273H) could still interact with and inhibit RPA *in vitro*. Therefore the ability to interact with RPA is not the sole mechanism by which p53 inhibits cellular DNA replication, because mutant p53 which can still bind RPA fails to inhibit replication. We hypothesize that p53 has to be capable of binding RPA *and* DNA to effectively inhibit the DNA replication apparatus. The transforming mutants of p53 do not bind DNA and so fail to be near origins of replication where the inhibition of RPA has the most profound effect on DNA replication. In this hypothesis, the ability to interact with RPA is still important for the replication inhibitory property of p53, and the prediction is that point mutant forms of p53 which can bind to DNA, but do not bind RPA, will have lost the growth suppression function.

This brief description is intended to highlight two issues that are fundamental to this application. (1) Mutational inactivation of p53 is a common feature in the initiation and progression of breast cancers. The interaction of a known replication factor RPA with a tumor suppressor protein p53, is a novel finding that should be investigated in greater detail. The interaction is likely to have important consequences for the mechanism of growth suppression by p53 and may provide a novel therapeutic target (RPA) for breast cancers with mutated p53. (2) Though p53 is mutated in up to 50% of breast cancers, a significant fraction have wild type p53. In these cancers p53 could be inactivated by interaction with a mutant or over-expressed cellular protein that either masks the transcriptional activation domain of p53, or inhibits its nuclear localization domain. Therefore, RPA p70, which interacts with both these domains of p53, could inactivate p53 and play a direct role in breast cancer tumorigenesis.

BODY

SPECIFIC AIMS FOR YEAR 1

1. Define the part of p53 involved in binding RPA and mutate it to obtain mutant forms of p53 which do not bind RPA. (Task 1)
2. Analyze the effect of these mutations on the transcriptional property of p53. (Task 2a)
3. Define the part of Rpa1 that binds p53. (Task 3, first half)

METHODS

Plasmid constructions for making deletions of p53. Deletions of p53 were made by PCR with the appropriate primers such that a fragment of DNA encoding the relevant portion of p53 was made and cloned into pGTK between BamHI and SalI sites. The resulting plasmid expressed the deletion fragment of p53 fused in frame to GST. The protein was expressed in a standard strain of E. coli, and the fusion protein purified by affinity on glutathione agarose beads. The point mutations that changed W53 to S, and F53 to S were also made by PCR with appropriate mutagenic oligonucleotides. In this case the mutant form of p53 was also cloned into a mammalian expression vector, pcDNA3, which expressed the mutant p53 (without any N terminal fusion) upon transfection in SaOs2 cells.

Plasmid constructions for making deletions of Rpa1. The deletion derivatives of Rpa1 were made as follows. p1-616 was the original phRPA1 clone obtained from Dr. T. Kelly where the RPA1 cDNA is cloned into pKS- between EcoRI sites such that the RPA1 gene is downstream from the T7 promoter. The EcoRI fragment was re-cloned into pKS+ in the reverse orientation to obtain prevRPA1. phRPA1 was cut with ClaI (sites in the untranslated region downstream from RPA1 and in the polylinker) and ligated to obtain phRPA1ΔCla . phRPA1 was cut with HindIII or with XhoI and ligated to obtain p1-219 and p1-307, respectively. phRPA1ΔCla was cut with HindIII and ligated to obtain pΔ223-411. phRPA1 was cut with BclI and EcoRV (in the polylinker), the ends filled in and ligated to obtain p1-491. prevRPA1 was cut with XbaI or with SspI and ligated to obtain p1-370 and p1-521 respectively. prevRPA1 was cut with XhoI and ligated to obtain p349-616, and phRPA1ΔCla cut with PstI and ligated to obtain p278-616.

Site-directed mutagenesis of RPA1. The Stratagene PCR Site-directed Mutagenesis Kit was used to generate pm1-616. Primer RPA70 S3V4 (5'AAATTCGGTGTCGACCTTCTCAGAGCGGTA CAATCC3'), is complementary to human RPA1 sequence 1555-1590 with underlined nucleotides changed from wild type sequence. Primer 1591-1621 is the same as the corresponding sequence of human RPA1. Primer 1591-1621 was 5'-phosphorylated and 15 pmole of each primer used for PCR on 0.5 pmole phRPA1 template DNA using Taq polymerase and Taq extender. The PCR cycling parameters are as follows: Segment 1: 1 cycle of 94°C 4 min, 50°C 2 min, 72°C min; segment 2: 8 cycles of 94°C 1 min, 56°C 2 min, 72°C 1 min; segment 3: 1 cycle of 72°C 5 min. By keeping the number of cycles low the chances of unintentional PCR induced mutation is decreased. The PCR reaction is thus used to create a linear DNA fragment corresponding to the whole phRPA1 plasmid except the mutations incorporated in the primers. 1 μl of DpnI (which cuts methylated bacterial DNA) and cloned Pfu DNA polymerase were added at 37°C for 30 min to simultaneously select against the parental template DNA and to polish the ends of products respectively. After heat inactivation of the enzymes (72°C for 30 min) the PCR product was circularized by ligation and transformed into E. coli. The resulting plasmids were screened for the incorporation of

the SalI site designed in the mutagenic primer, and candidate plasmids sequenced to confirm the mutation and rule out adjoining secondary mutations.

In vitro transcription-translation and binding assays. Radio labeled protein was synthesized with 35S methionine and the Promega TnT Coupled *in vitro* transcription-translation kit. For measuring the binding of Rpa1 to p53, the translation mixes were diluted 1:10 with NETN containing 50 mM NaCl and 10 mg/ml Blotto. After incubation for 1 hr at 4°C with glutathione agarose beads coated with approximately 1 μg of GST (glutathione-S-transferase) or GST-p53, the beads were washed with 4x1 ml NETN (50 mM NaCl). Bound proteins were eluted by boiling the DNA-cellulose pellet in Laemmli's buffer, electrophoresed on 15% SDS-polyacrylamide gel and visualized by fluorography in 1M Sodium salicylate.

RESULTS

Sub-domains of p53 synergize to give strong RPA binding: GST fusion proteins containing various fragments of p53 were generated, bound to glutathione agarose beads and their ability to bind RPA examined by affinity chromatography (Fig. 1). We have shown earlier that two domains of p53, N2 (amino acids 2-121) and 5C (amino acids 289-393), could independently bind RPA. Amino acids 2-71 of p53 (a domain) had equivalent RPA binding activity as 2-121 (data not shown). However amino acids 2-45 or 46-71 (sub-domains) showed much reduced RPA binding activity. Similarly at the C terminal end, while 289-356 had significant RPA binding, 289-330 or 331-356 did not have significant RPA binding. Ten times as much of each GST-sub-domain protein (e.g. GST 2-45 or GST 46-71) were compared to GST-domain protein (GST 2-71) in their ability to bind RPA. The binding activity of each sub-domain was less than one-tenth that of the corresponding domains. Thus the better binding of RPA by a domain (e.g. 2-71) is probably not a simple summation of RPA binding by each of the sub-domains (e.g. 2-45 and 46-71). Similar results were obtained with 289-356 (data not shown). It is unlikely that in two separate instances the absence of RPA binding by the sub-domains is due to the RPA binding site spanning the site of division. The alternative explanation is that weak RPA binding sites in each of the sub-domains synergize to produce the strong binding activity of the corresponding domain.

Further mapping of the RPA binding site of p53 (see below) suggests that amino acids 48, 49, 53 and 54 are important for the interaction. Therefore, the failure of 46-71 to bind RPA may be because the fusion with GST at amino acid 46 inadvertently changes the structure of the RPA binding site or even truncates it.

Aromatic amino acids in a sub-domain of p53 are important for RPA binding: The transcriptional trans-activator VP16 has been shown to interact with RPA, and a phenylalanine to proline mutation in VP16 shown to diminish RPA binding. Reasoning that a similar mechanism of interaction occurred between RPA and p53, point-mutations were made in p53 which changed two adjoining aromatic amino acids, tryptophan and phenylalanine (residues 53-54) in one of the sub-domains of N2 to serines (W53S-F54S). This GST fusion protein did not bind RPA (data not shown). Several other point mutations have also been made in the N terminal part of p53 in the laboratory of Dr. A. Levine, and a representative collection of these and W53S-F54S were engineered into GST-p53 fusion proteins and their RPA binding activity determined (Fig. 2). The results demonstrate that the aromatic residues W53 and F54 are important for RPA binding. Mutations in amino acids 48-49 (D48H-D49H) also decreased RPA binding, suggesting that negatively charged amino acids near the hydrophobic residues at 53-54 were important for RPA binding. The mutations which changed amino acids 22-23 of p53 (L22Q-W23S) affect its ability to activate transcription [19], but did not affect its ability to bind RPA. Thus although in the herpesvirus transcriptional activator VP16 the same amino acid (F442) is important for both interaction with RPA and activation of

transcription, this is not the case with p53. Therefore it seemed likely that we can separate the trans-activation and RPA binding functions of p53 with appropriate point-mutations.

RPA from crude cell extracts does not bind to the 5C domain of p53: The W53S-F54S mutation in p53 produced a significant decrease in the binding of RPA from crude cell extracts (S100 extracts). This result was unexpected because the C terminal 5C domain of p53 had also been shown to interact with purified RPA. One explanation could be that 5C is unable to bind RPA from S100 extracts. When tested directly, we found that while N2 could bind RPA from both purified fractions and from cell extracts, 5C could only bind RPA from the former (Fig. 3). Although we do not know why the 5C part of p53 does not bind RPA from crude cell extracts, the above observation explains how we obtained a mutant form of p53 which loses the ability to bind RPA from cell extracts by making mutations only in the N2 domain of p53. This approach has the added advantage of leaving intact the dimerization and nuclear localization functions in the 5C domain which are essential for growth suppression.

Specific interactions of the C terminal portion of p53 with other products in the S100 extract could be responsible for the failure of p53, 5C, to interact with RPA in the crude extract. However, pre-incubation of GST-5C with S100 extract, washing off unbound proteins and then adding pure RPA did not block the binding of RPA to 5C. In the reverse experiment, pure RPA was added to S100 extract and then asked to bind to 5C. Again the association of pure RPA to 5C was not affected by the S100 extract. Hence at the moment we do not understand why the RPA in the S100 extract fails to bind to 5C. The best hypothesis is that the RPA present in the S100 extract is in a complex with some cellular protein which prevents it from interacting with 5C.

Transcription activation by p53 mutants: To test the transcription activation properties of these p53 molecules, a transient transfection assay was done (Fig. 4). Plasmids expressing p53 and various mutant derivatives were co-transfected into SaOs2 cells with a reporter plasmid, 6FSVCAT, which has six consensus p53 binding sites cloned upstream from a CAT gene. Only the L22Q-W23S mutation of p53 significantly affected transcription activation by p53, although it still retained five-fold activation over vector control. D48H-D49H, W53S-F54S and D61H-E62K mutant forms of p53 retained 50-100% of transcriptional activity compared to wild-type p53. In D48H-D49H and W53S-F54S we had versions of p53 with near wild-type trans-activation but significantly diminished RPA binding activities.

Region of Rpa1 required for binding p53. We have reported that RPA bound to p53 fails to bind single-stranded DNA [18]. One explanation could be that the overlapping regions of Rpa1 are required to bind the two ligands, so that the ligands are mutually exclusive. To determine if this was the case, we used the deletion derivatives of Rpa1 to map the region required to bind to p53. Rpa1 and deletion derivatives were synthesized in vitro and bound to glutathione agarose beads coated with either glutathione S transferase (GST) or GSTp53 (Summarized in Fig. 5). In contrast to the binding region of ssDNA or Rpa2, the binding region of p53 was difficult to map from the deletions of Rpa1. A small C terminal deletion increased binding to p53 (1-616 v. 1-521) suggesting the presence of an inhibitory domain at the extreme C terminus. Due to the weak binding of 1-370 the p53 binding region may already be affected by this deletion. The inability of 278-616 to bind p53 also suggests that residues 1-278 is essential for p53 binding.

Overall, the deletion derivatives that contained the 1-521 region of Rpa1 could bind p53 The existence of inhibitory (521-616) domains which influence binding to GST-p53 was unexpected.

The putative zinc finger of Rpa1 is dispensable for binding single-stranded DNA, p53 or Rpa2. A putative C4-type zinc finger motif was noted at position 481-503 of human Rpa1, which is evolutionarily conserved in yeast Rpa1. Since 1-491 could weakly bind p53, the zinc finger is also dispensable for binding p53. To confirm whether the zinc finger was important for binding p53, point-mutations were made in the Rpa1 cDNA which changed the 2 C terminal cysteines of the putative zinc finger to serines (m1-616). This point-mutated form of Rpa1 was synthesized in vitro and its ability to bind p53 measured by pull-down with GST-p53 beads. The mutated form of Rpa1 associated with p53 as effectively as wild-type Rpa1 (data not shown). Therefore, the putative zinc finger is not required for binding p53.

FIGURE LEGENDS

Fig. 1 Binding of RPA by GST fusion proteins containing the indicated portions of p53 (e.g. amino acids 289-356 etc.) GST: glutathione S transferase without any p53 fusion. 0.1 input: one-tenth of the input RPA mixed with glutathione beads containing equal quantities of the indicated GST proteins. After incubation and washing the RPA bound on the beads is visualized as described in Ref. 18. On the right hand side we show that even when one-tenth as much 2-71 (third lane from right) is used as 2-45 or 46-71, the association of RPA is stronger with 2-71.

Fig. 2 Binding of RPA from purified RPA preparation (RPA) or from crude cell extract (S100) by GST fused to amino acids 2-117 of p53 (N2) or amino acids 289-393 of p53 (5C). p70 and p34 are Rpa1 and Rpa2 respectively. The rest is as in Fig. 1.

Fig. 3 Binding of RPA by GST fusion proteins containing mutant alleles of p53 with the indicated amino acids mutated by site-directed mutagenesis. wt p53: wild type p53. 61-62: D61H-E62K where the aspartic acid (D) at position 61 is changed to histidine (H) and so on, 53-54: W53S-F54S, 48-49: D48H-D49H, 22-23: L22Q-W23S, 14-19: L14Q-F19S. The rest is as in Fig. 1.

Fig. 4 Transcription transactivation by various alleles of p53 assayed by transient transfection of plasmids expressing p53 with FSVCAT, a plasmid containing the CAT gene under control of a p53 responsive promoter, into p53 null SaOs2 and H1299 cells. cDNA3: vector alone, wt: cDNA3 containing wild-type p53. The rest as in Fig. 3.

Fig. 5 Summary of binding of Rpa1 with Rpa2, p53 and single-stranded DNA cellulose (the last only at 0.5M NaCl). Binding of Rpa1 and derivatives to GST-p53 was measured as described in the text. The Rpa1 and deletion derivatives are represented by rectangles of appropriate length and the amino acids at the limits of the deletions indicated. Binding of the same derivatives to single-stranded DNA and to Rpa2 are not the subject of this grant, but are indicated for comparison with the p53 binding region. +: binding the same as wild-type Rpa1 (1-616), with ++ indicating binding better than wild-type, and +/- indicating binding less than wild-type.

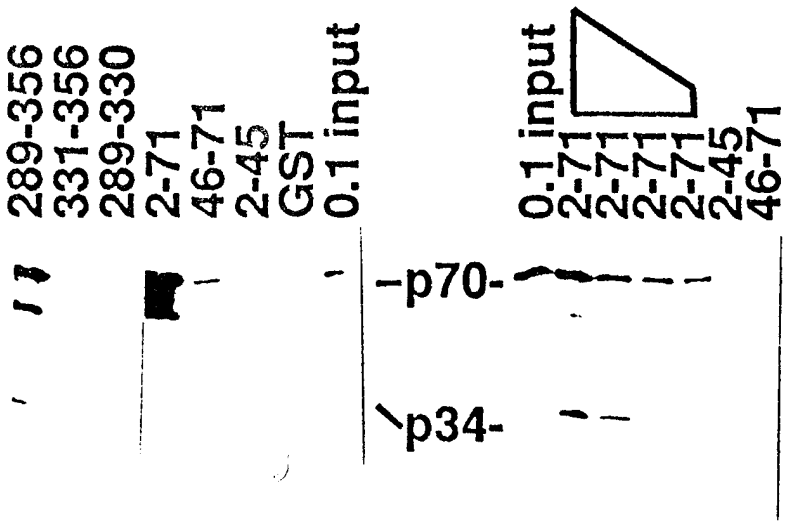
289-356
331-356
289-330
2-71
46-71
2-45
GST
0.1 input
0.1 input
2-71
2-71
2-71
2-71
2-45
46-71
p70-
p34-

Figure 1

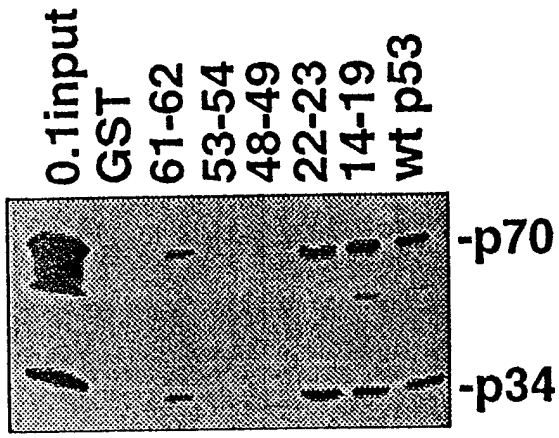

Figure 2

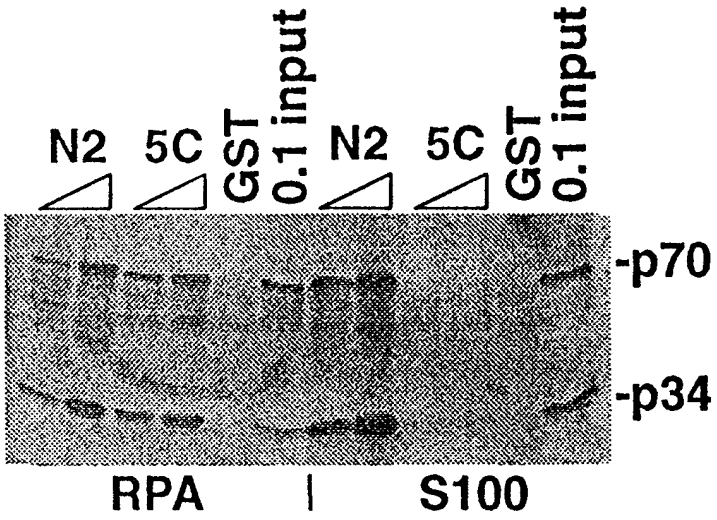

Figure 3

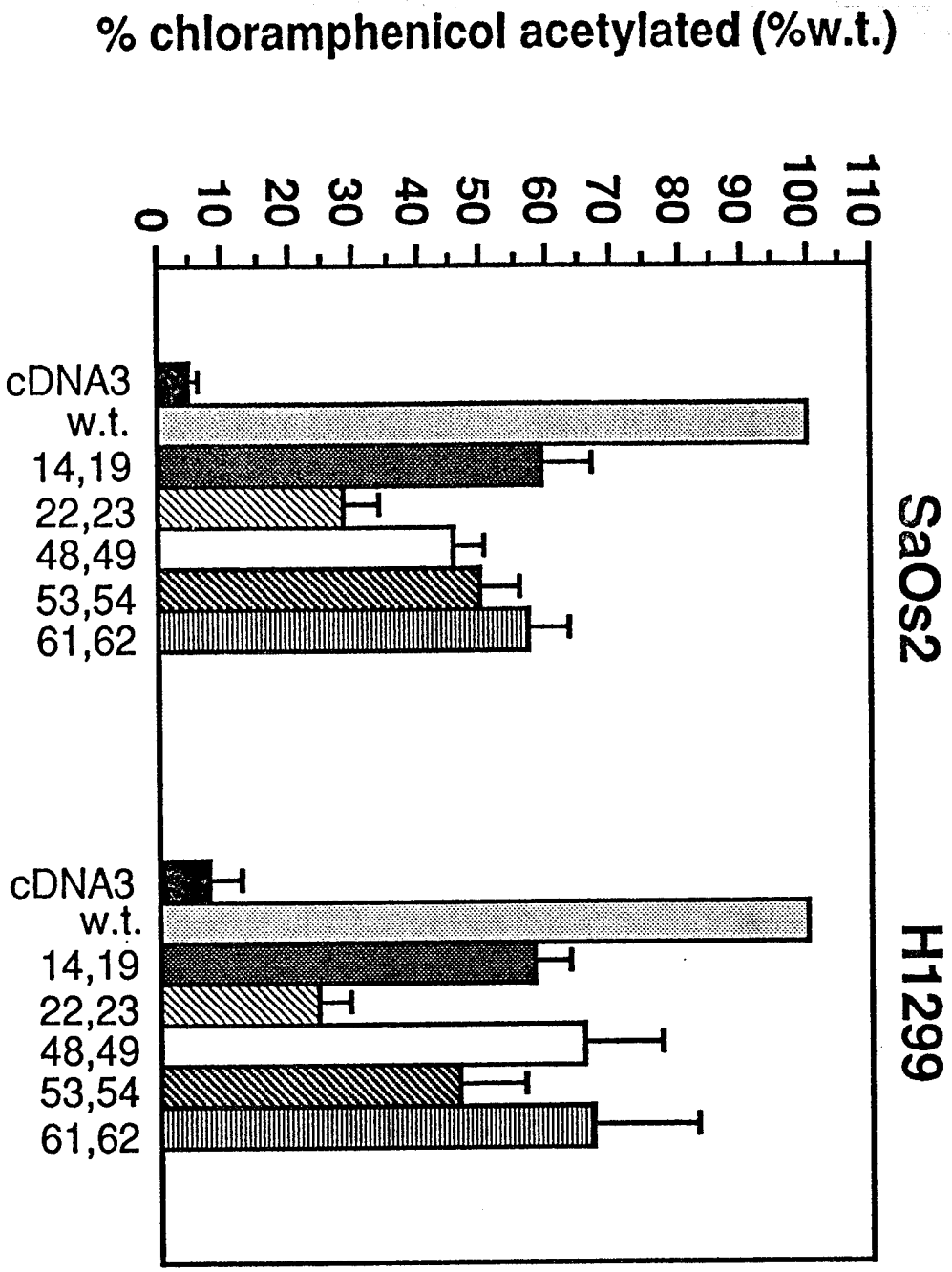
% chloramphenicol acetylated (%w.t.)
0
10
20
30
40
50
60
70
80
90
100
110
cDNA3
w.t.
14,19
22,23
48,49
53,54
61,62
SaOs2
cDNA3
w.t.
14,19
22,23
48,49
53,54
61,62
H1299

Figure 4

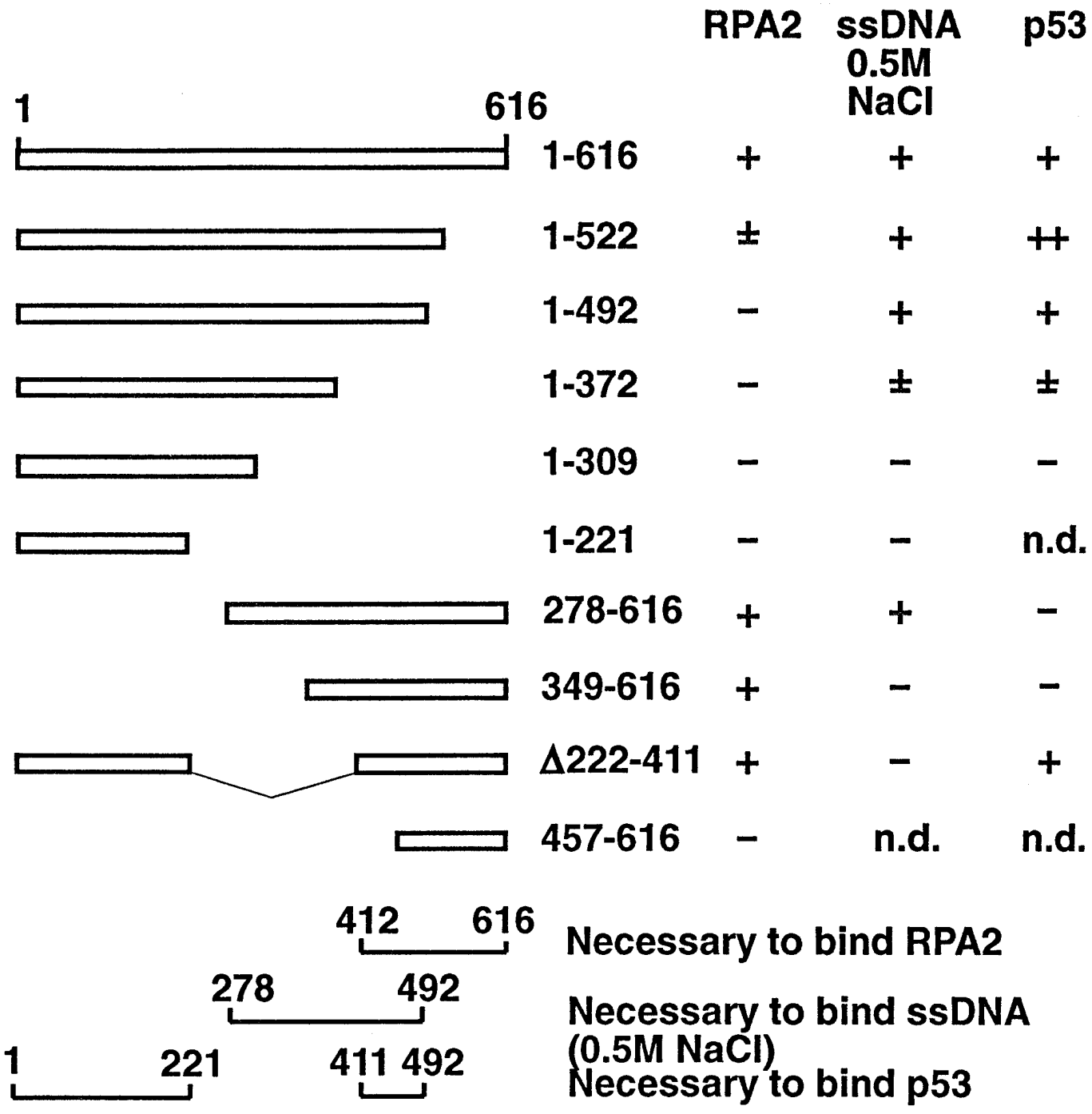
RPA2
ssDNA 0.5M NaCl
p53
1
616
1-616 + + +
1-522 ± + ++
1-492 – + +
1-372 – ± ±
1-309 – – –
1-221 – – n.d.
278-616 + + –
349-616 + – –
Δ222-411 + – +
457-616 – n.d. n.d.
412 616
Necessary to bind RPA2
278 492
Necessary to bind ssDNA (0.5M NaCl)
1 221
411 492
Necessary to bind p53

Figure 5

CONCLUSIONS

Relatively short peptides with aromatic amino acids surrounded by negatively charged residues produce RPA binding activity when repeated several times in a protein. The degenerate nature of this RPA binding signal probably accounts for the large number of proteins reported to bind RPA. Since similar bulky hydrophobic residues in the context of negatively charge amino acids are also essential for transcriptional trans-activation, this observation may explain why several transcriptional activators are able to bind RPA. Potentially, this could be a common feature of several protein-protein interactions, although at present it is restricted to interactions between transcription/replication regulatory proteins, and proteins of the basal transcription or replication apparatus. The recently reported interaction between the DNA repair protein XP-G and RPA also uses a negatively charged part of XP-G, and it is possible that the same mode of interaction is involved [20].

Bulky hydrophobic residues in the N terminal region of p53 have already been implicated in transcriptional trans-activation and in interactions with the TATA binding protein and viral and cellular oncogenes E1b and mdm2 respectively [19]. Although our results suggest that at least for RPA binding the interaction depends on relatively degenerate sequences, there must be some specificity to the modules that interact with transcriptional factors versus the ones that interact with RPA because we were able to create point mutation which affected RPA binding but not trans-activation and vice-versa. The exact source of this specificity will become more clear when the interaction domains of the other partners (RPA, transcriptional co-activator, E1b or mdm2) in these interactions are defined.

Peptides of varying lengths could be multimerized to produce RPA binding. Therefore, there do not appear to be strict structural constraints on the interactor modules, because each of the multimers have different distances between the aromatic residues, between the acidic residues and between the aromatic and acidic residues. We think that the interactor modules are unstructured, and may be induced into a more defined structure when the other partner is bound. This "induced fit" hypothesis also leaves room for specificity of interaction depending on the structure of the other partner in the interaction. One could speculate that the minimal requirements of the other partner would be to have a distribution of bulky hydrophobic residues surrounded by positively charged residues, so that hydrophobic and electrostatic interactions would stabilize the interaction.

L22Q-W23S showed decreased transcription activation but wild-type RPA binding indicating that transcriptional trans-activation function is not required for RPA binding. On the other hand, D48H-D49H and W53S-F54S showed wild-type transcription activation and lost RPA binding, confirming that the RPA binding site of p53 is different from its transcription trans-activating site. It will be interesting to determine how these mutation of p53 affect (a) its ability to repress transcription, (b) ability to suppress growth of cells, and (c) ability to induce apoptosis.

Scrutiny of the Rpa1 deletions shows that association with p53 did not correlate with binding to single-stranded DNA or to Rpa2. There was a derivative which bound p53 well but not single-stranded DNA (349-616) and others which bound both well (1-521). Likewise there was an Rpa1 derivative which bound Rpa2 well but not p53 (Δ223-411), two that did the reverse (1-521 and 1-491) and another that bound both well (349-616). This confirms our previous observation that the Rpa1-p53 interaction did not require Rpa2 or single-stranded DNA [18]. The failure of 278-616 to bind p53 puts the N terminal limit of the p53 binding domain N terminal to amino acid 278. The C terminal limit of the minimal p53 binding domain is probably between residues 491 and 521. We have also defined the minimal domains of Rpa1 that are required to bind Rpa2 and single-stranded DNA, and the results suggest that the p53 binding site significantly overlaps with the sites for binding DNA, but not with the site for binding Rpa2. This

explains why p53 bound RPA remains as a holocomplex (Rpa1+Rpa2+Rpa3) but fails to bind single-stranded DNA.

The ability to divide the Rpa subunits into sub-domains required for essential activities (holocomplex formation and DNA binding) opens the way toward screening for point mutations in Rpa1 which can still form the holocomplex, and bind DNA, but cannot bind p53 or other transcriptional activators. Should such a mutant form of Rpa1 be obtained, we shall be able to test the importance of the p53-Rpa1 interaction on RPA function in replication, repair and recombination or the regulation of these processes by the reported interactions with p53, transcriptional activators and DNA repair proteins.

Overall the project is progressing on schedule and the reagents generated will help us answer the questions we set out to address in the original grant proposal. Our lab has made additional progress on other aspects of p53 function and RPA. Specifically, we have demonstrated that a transcriptional target of p53, the p21 protein interacts directly with another DNA replication factor, PCNA, and in so doing inhibits DNA replication [21]. This could be a novel mechanism by which p53 keeps the DNA replication and repair apparatus in control in normal mammary epithelial cells. We have also discovered a homolog of the middle subunit of RPA, tentatively named Rpa4 [22]. We will at a future date test whether this novel form of RPA composed of Rpa1+Rpa4+Rpa3 has similar p53 binding properties as the authentic RPA (Rpa1+Rpa2+Rpa3).

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